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CARDIOPROTECTIVE EFFECT OF NICORANDIL IN HTK SOLUTION DURING THE COLD STORAGE OF ISOLATED HEARTS

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Abbreviations: ADP; adenosine diphosphate, AF; aortic flow, AMP; adenosine monophosphate, ATP; adenosine triphosphate, CF; coronary flow, c-GMP; cyclic guanylic acid, CO; cardiac output, CPK; creatine phosphokinase, EC; adenylate energy charge, HR; heart rate, i.p.; intraperitoneally, K_{ATP}; ATP sensitive potassium, KHB; Krebs-Henseleit bicarbonate buffer, LA; left atrium, L; Langendorff, LV; left ventricle, NCR; Nicorandil, RPP; rate pressure product, SP; systoric pressure, SR; sarcoplastic reticulum, TAN; total adenine nucleotides, UW; University of Wisconsin, W; working

Abstract

We compared the efficacy of using HTK solution with that of University of Wisconsin (UW) solution for heart preservation in an isolated rat heart preparation. Nicorandil (NCR) exerts its action as an ATP-sensitive potassium channel opener at low extracellular potassium concentrations, and HTK solution has a low potassium concentration. Therefore, we also investigated the efficacy of using HTK solution with NCR following 12-hour preservation. Hearts isolated from male Wistar rats were mounted on a Langendorff apparatus to estimate baseline aortic flow (AF), coronary flow (CF), cardiac output (CO), heart rate (HR), systolic pressure (SP), aortic mean pressure, and the rate-pressure product (RPP). The hearts were divided into 4 groups: Group 1, 8-hour storage in UW solution; Groups 2 and 3, 8 or 12-hour storage in HTK solution, respectively; Group 4, 12-hour storage in HTK solution with NCR. They were arrested and stored at 4°C in each preservation solution. Following storage, they were reperfused and post-preservative function was measured to assess cardiac functional recovery. Concentrations of CPK, troponin-T, and lactate in the coronary perfusate were measured. Frozen tissue samples from Groups 3 and 4 were analyzed for adenylate content and cyclic-GMP. The myocardial water content was also measured. The recovery of AF, CF, CO, SP, and RPP in Group 2 were significantly improved compared with that in Group 1 ($p<0.05$). The recovery of AF, CF, CO and HR in Group 4 were significantly better than in Group 3 ($p<0.05$). CPK leakage in Group 2 and Troponin-T leakage in Group 4 were significantly reduced ($p<0.05$ vs Group 1 and 3, respectively). Total adenine nucleotides and the adenylate energy charge in Group 4 were well-sustained ($p<0.05$ vs Group 3). These results suggest that HTK solution is more effective than UW solution for cardiac preservation, and that NCR provides better protection still more.

Introduction

Following the introduction of University of Wisconsin (UW) solution, the preservation times of solid organs have been markedly extended, both in animal and clinical experiments (1-6). However, the generally accepted time limit for safe cold storage for clinical heart trasplantation remains only 4 to 6 hours (7). HTK organ preservation solution was initially developed by Bretschneider and coworkers as a cardioplegic solution (8). The protective effect of HTK solution is based on the high buffering capacity provided by histidine, restricting tissue acidosis induced by ischemia. HTK solution has been reported to be effective in experimental kidney transplantation, and a clinical trial is currently underway (9,10). The purpose of this study is to assess the efficacy of HTK solution as compared with UW solution in experimental heart preservation .

Nicorandil (NCR), nitrate and K_{ATP} channel opener, has a remarkable coronary vasodilating effect and is being used as a theraputic drug for angina (11-14). Moreover, it has been reported that NCR has cardioprotective effects, through the attenuation of ATP depletion (15), and protection against free radicals (16). Therefore, we also investigated the effect of NCR on myocadial protection during simple cold storage.

Materials and Methods

Isolated rat heart preservation

Animal experimentation was performed in accordance with institutional guidelines of the Guide for the Care and Use of Laboratory Animals published by the U.S. Department of Health and Human Services. Male Wistar rats (250 to 350 g) were systemically heparinized [500 IU intraperitoneally (i.p.)], and anesthetized with sodium pentobarbital (65 mg/kg i. p.). The heart was excised and immediately immersed in Krebs-Henseleit bicarbonate buffer (KHB), consisting of (mmol/L): NaCl (118), KCl (4.7), $MgSO_4$ (1.2), KH_2PO_4 (1.2), $CaCl_2$ (2.5), $NaHCO_3$(25.0), and glucose (11.0) at 37°C. Then, it was mounted on a Langendorff apparatus (IPH-W, Labo Support, Osaka,

Japan) via the aorta, and perfused with KHB solution at a constant pressure of 65 mmHg for 3 minutes in Langendorff mode (L-mode). KHB solution was filtered (48 μm), equilibrated with 95% O_2 and 5% CO_2, and maintained at 37°C. During preparation, care was taken to cannulate the excised hearts rapidly to minimize the ischemic time. Following cannulation of the left atrium (LA) via the pulmonary vein, the heart was switched to working mode (W-mode) with an LA perfusion pressure of 10 mmHg and an afterload of 60 mmHg. Baseline function was determined following 2 minutes of W-mode. Measurements were as follows. aortic flow (AF), coronary flow (CF), cardiac output (CO), heart rate (HR), systolic pressure (SP), aortic mean pressure (MP) and rate pressure product (RPP, HR x max SP). Following measurement of pre-preservative cardiac function, the heart was switched back to L-mode.

Experimental protocol

Experimental protocol is shown in Figure 1. The hearts were divided into four groups (n=5 to 8 hearts per groups): Group 1 (n=7), 8-hour storage in UW solution; Group 2 (n=8), 8-hour storage in HTK solution; Group 3 (n=5), 12-hour storage in HTK solution; Group 4 (n=6), 12-hour storage in HTK solution with NCR 10^{-4} M. The hearts in all groups were arrested by administration of each preservation solution (60 mL/kg at 4°C) via the aortic cannula at a pressure of 60 mmHg. Then, they were stored in each preservation solution (30mL) at 4°C. Table 1 shows the compositions of the preservation solutions . Following cold storage, they were mounted on a Langendorff apparatus again, and reperfused for 15 minutes on L-mode. Then, they were switched to W-mode. The coronary perfusate was collected following 10 minutes of W-mode reperfusion to evaluate for lactate, CPK, and troponin-T in each preservation group. Lactate was measured according to an enzymatic method, CPK according to Ultra Violet method and troponin-T according to an enzyme immunoassay method. Cardiac functional recovery of the stored heart was measured at the end of 25 minutes of W-mode

reperfusion. Following evaluation of the stored heart, LV dp/dt (mmHg/sec) was measured by puncture via the left ventricular apex. A ventricular specimen was weighed immediately, dried at 80°C to constant weight, and reweighed following 24 hours. Water content was computed using the following formula: water contents (%) = (wet weight - dry weight)/ wet weight x 100.

Biochemical analysis

Frozen myocardial samples in liquid nitrogen just after storage in the solution of Group 3 and Group 4 were analyzed for adenylate content and cyclic-GMP (c-GMP).

Adenylate content

Frozen myocardium was centrifuged (11,000 g for 5 minutes at 4°C) before removal of the supernatant and neutralization with potassium hydroxide (2 mol/L). Aliquots (20 μl) were taken for analysis by high performance liquid chromatography. The values obtained were used to calculate the following indices of myocardial energy status: total adenine nucleotides (TAN) = ATP + ADP + AMP and adenylate energy charge = (0.5 ADP + ATP)/(ATP + ADP + AMP).

Cyclic-GMP

Frozen myocardium was homogenated 1ml of 0.1 N HCl and centrifugal at 25000g for 15 min. The c-GMP content in the supernatant was measured for radioimmunoassay.

Chemicals

Nicorandil was obtained from Chuugai Pharmaceutical. Co. LTD, Japan.

Statistical analysis

All results are expressed as the means ± SE. A statistical analysis was performed by Student's unpaired t test. A p value of less than 0.05 was considered statistically significant.

Results

Baseline cardiac function

The mean values of baseline cardiac function (n=26), measured prior to preservation, were as follows; 45.7± 2.7 ml/min of AF, 15.7±0.8 ml/min of CF, 64.2 ±1.3 ml/min of CO, 228.8 ±10.7 beats/min of HR, 108.3 ±3.6 mmHg of SP and 23347 ±344 of RPP.

Cardiac functional recovery

Table 2 shows the recovery of hemodynamic data on the heart for 8 or 12 hours of preservation [(A), absolute values of post-preservative cardiac function; (B), percentage values to the pre-preservative baseline function). Following 8 hours of preservation , the recovery of AF, CF, CO, SP, and RPP in Group 2 was significantly increased compared with that in Group 1. Following 12 hours of preservation , the recovery of AF, CF, CO and HR in Group 4 was higher than that in Group 3 ($p<0.05$). There were no significant differences in SP, RPP or LV dp/dt between Group 3 and 4.

CPK, Lactate and Troponin-T leakage

There was a significant decrease in CPK leakage in Group 2, compared with Group 1 (Table 3). Troponin-T leakage in Group 4 decreased significantly, compared with Group 3 (Table 3). There were no significant differences in lactate leakage among the four groups.

Adenylate contents and cyclic-GMP

TAN and EC were maintained better in Group 4, compared with Group 3 ($p<0.05$) (Table 4). There were no significant differences in c-GMP levels.

Water content

The tissue water content was 83.2 ±0.4% in Group 1, 82.6 ±0.5 % in Group 2, 83.4 ± 0.3% in Group 3, and 82.9 ±0.8% in Group 4. There were

no statistically significant differences among the groups.

Discussion

A "universal " preservation solution would be suitable for preservation of all organs, and would increase the simplicity of the overall harvesting procedure. Numerous preservation solutions have been proposed and experimentally evaluated in response to the need for extending the safe limit of organ preservation. University of Wisconsin (UW) solution has been demonstrated to provide significant improvement in organ function after the extended preservation of kidneys, livers and pancreases (1-6). Several studies have compared UW solution with other preservation solutions for the heart, and have found it superior (17,18), however the clinically safe time limit remains only 4 to 6 hours (7). HTK solution has been reported to be effective in kidney and liver transplantation (9,10,19,20). However, it has not been as extensively tested for use with the donor heart (21). Hendry et al. have reported better recovery of human right atrial trabeculae contracting isometrically in vitro after preservation with HTK solution, compared with UW solution (22).

In this study, we examined the cardiac functional recovery of the whole heart in a working mode where cardiac function could be evaluated more precisely, and found a better recovery of cardiac function and less leakage of CPK in the group preserved with HTK solution, compared with that preserved with UW solution, following an 8- hour storage. During cold storage, intracellular pH drops as H+ and lactate accumulate and ATP is depleted, which causes dysregulation of cellular homeostasis and leads to cell injury (23). The suppression of ischemia-induced tissue acidosis and sustenance of cytosolic ATP pools by the high buffering capacity provided by histidine (20) might have lead to the better cardiac functional recovery with HTK solution in our study.

During cold storage in the presence of a low intracellular pH, a high potassium concentration has been shown to lead to myocardial damage (24).

Another study has also revealed that a high potassium concentration might be harmful to the endothelium (25). Therefore, HTK solution, which has a low potassium concentration, might provide excellent protection. The much higher potassium concentration in UW solution is than in HTK solution might not be suitable for cardiac preservation.

In UW solution, raffinose, lactobionate, and colloid hydroxyethyl starch suppress the parenchymal cell swelling induced by hypothermia. In HTK solution, histidine, apart from being a buffer, appears to be effective in preventing cell swelling. The impermeability of UW solution has been reported to be superior to that of HTK solution for long-term preservation beyond 24 hours (20). However, considering graft viability, it may not be possible to preserve a heart for such a long time. In the present study, the tissue water content following an 8- or 12-hour preservations revealed no statistically significant differences with the preservation solution. The inhibitory effect on cell swelling in HTK solution might be as good as that in UW solution, during short-period preservation as in this study.

Recently, Nicorandil (NCR) has drawn attention for its cardioprotective effect in ischemia and reperfusion injury through a dual mechanism of action involving nitrate effects via activation of guanylate cyclase, and the opening of K_{ATP} channels (11-14). Therefore, in this study, we added NCR to HTK solution, preserved hearts in it for 12 hours, and found much better recovery of cardiac function, and a significant decrease in troponin-T leakage following 12-hour storage, compared with that without NCR. The total adenine nucleotides and adenylate energy charge of hearts preserved in HTK solution with NCR were better sustained than those of hearts preserved in HTK solution without NCR.

During cold storage, energy is used in the preserved myocyte to maintain intracellular homeostasis, particularly that of calcium. As energy stores are depleted, the myocyte can no longer control its internal homeostasis, and the sarcoplasmic reticulum and mitochondria can no longer maintain their sequestration of calcium. Calcium is then released in the cytosome to

contracture by interacting with tropomyosin, creating a high-affinity actin-myosin state. The actin and myosin interact causing a progressive and irreversible contracture of the heart (26,27). At reperfusion, intracellular Ca^{2+} influx is increased further and myocardial damage is accelerated. NCR, which activates the K_{ATP} channels may decrease the action potential duration and attenuate membrane depolarization. These effects may lead to a decrease in the free cytosolic calcium concentration, a rapid loss of contractile activity, and a reduced level of adenosine triphosphate (ATP) utilization, which prevent myocardial injury during preservation and reperfusion (P/R).

The action of NCR as the K_{ATP} channel opener has been reported to be more effective when the extracellular potassium concentration is below 24 mM (28). The potassium concentration in HTK solution is 10 mM, and that in UW solution is 125 mM. Therefore, in this study, we added NCR just to HTK preservation solution and found a better cardiac functional recovery, following P/R.

In this study, we measured myocardial c-GMP as a marker of nitric oxide, since NCR has a nitrate action in addition to K_{ATP} channel opening action. Myocardial c-GMP might increase in hearts stored in HTK solution with NCR, because of the nitrate effect. However, there were no significant differences in myocardial c-GMP concentrations between the hearts stored in HTK solution with NCR and those stored in HTK solution without NCR. In our study, the coronary flow of hearts preserved in HTK solution with NCR increased significantly, compared with those preserved in HTK solution without NCR. During cold storage, thus, the K_{ATP} channel effect of NCR appears to be more prominent than its nitrate effect.

In conclusion, HTK solution is much more effective than UW solution for heart preservation, and HTK preservation solution with NCR provides further protection, which may lead to better techniques of heart preservation for transplantation.

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Table 1. Composition of the preservation solutions

HTK		UW	
NaCl	15	KH_2PO_4	25
KCl	9	KCl	5
$MgCl_2$	4	Adenosine	5
K-ketoglutarate	1	Glutathione	3
Histidine	180	$MgSO_4$	5
Histidine HCl	18	Raffinose	30
Mannitol	30	Allopurinol	0.01
		K-Lactobionate	100
		Pentastarch	5%

(m mol/L)

TABLE 2. (A) Cardiac Functional Recovery

Group	Time (hours)	AF (ml/min)	CF (ml/min)	CO (ml/min)	HR (beats/min)	SP (mmHg)	RPP ($x10^3$)
1. UW (n=7)	8	20.4±2.8	8.3±1.1	30.8±1.9	207±24	76.3±2.2	16.8±0.5
2. HTK (n=8)	8	33.1±4.1*	14.4±0.9*	49.1±3.7*	214±18	98.7±6.7*	20.4±1.2*
3. HTK (n=5)	12	3.5±0.5	6.6±0.5	9.8±1.2	138±7	66.3±6.6	10.8±1.0
4. NCR (n=6)	12	6.0±1.2#	11.2±0.7#	18.4±3.0#	196±14#	67.2±3.4	12.2±1.6

(B) Cardiac Functional Recovery (%)

Group	Time (hours)	AF (%)	CF (%)	CO (%)	HR (%)	SP (%)	RPP (%)
1. UW (n=7)	8	47.4±4.2	57.4±6.2	49.7±2.4	91.6±0.8	77.3±1.3	71.0±1.8
2. HTK (n=8)	8	71.5±8.6*	87.8±5.8*	78.1±5.9*	92.5±0.8	90.1±4.6*	83.6±4.6*
3. HTK (n=5)	12	7.2±1.0	40.9±3.1	16.0±1.8	71.2±2.6	58.7±5.6	43.0±3.9
4. NCR (n=6)	12	12.4±2.6#	66.6±10.9#	29.7±4.7#	82.9±5.7#	56.5±2.5	47.2±4.9

Data are expressed as mean±S.E. The hemodynamics of the pre-preservative hearts were: AF(45.7±2.7 ml/min), CF(15.7±0.8 ml/min), CO(64.2±1.3 ml/min), HR(229±11 beats/min), SP(108±3.6 mmHg), and RPP(23347±344).

*$p<0.05$ vs Group 1, #$p<0.05$ vs Group 3

NCR, HTK solution with Nicorandil 10^{-4} M

AF, aortic flow; CF, coronary flow; CO, cardiac output; HR, heart rate; SP, systolic pressure; RPP, rate pressure product

Table 3. CPK, Troponin-T and Lactate leakage

	Time (hours)	CPK (IU/min/g)	Tn-T (ng/min/g)	Lactate (mg/min/g)
1. UW (n=7)	8	0.06 ± 0.02	19.7 ± 3.4	10.3 ± 0.7
2. HTK (n=8)	8	0.01 ± 0.00 *	26.7 ± 9.8	6.5 ± 2.1
3. HTK (n=5)	12	0.3 ± 0.3	37.5 ± 8.9	10.1 ± 1.7
4. NCR (n=6)	12	0.01 ± 0.00	8.9 ± 2.0 ※	13.4 ± 2.1

Values are mean ± S.E. ＊p<0.05 vs Group 1 ※p<0.05 vs Group 3

CPK: creatine phosphokinase, Tn-T: troponin-T, NCR: HTK solution with Nicorandil 10^{-4} M

Table 4. Adenylate contents and cyclic-GMP

	Time (hour)	TAN (μ mol/g)	Energy Charge	c-GMP (p mol/g)
Group 3 (n=5)	12	8.1 ±0.5	0.22 ±0.03	13.3 ±0.3
Group 4 (n=6)	12	14.9* ±0.6	0.53* ±0.05	13.0 ±1.0

Values are mean ± SE. *$p < 0.05$ vs Group 3

TAN: Total adenine nucleotides, c-GMP: cyclic guanylic acid

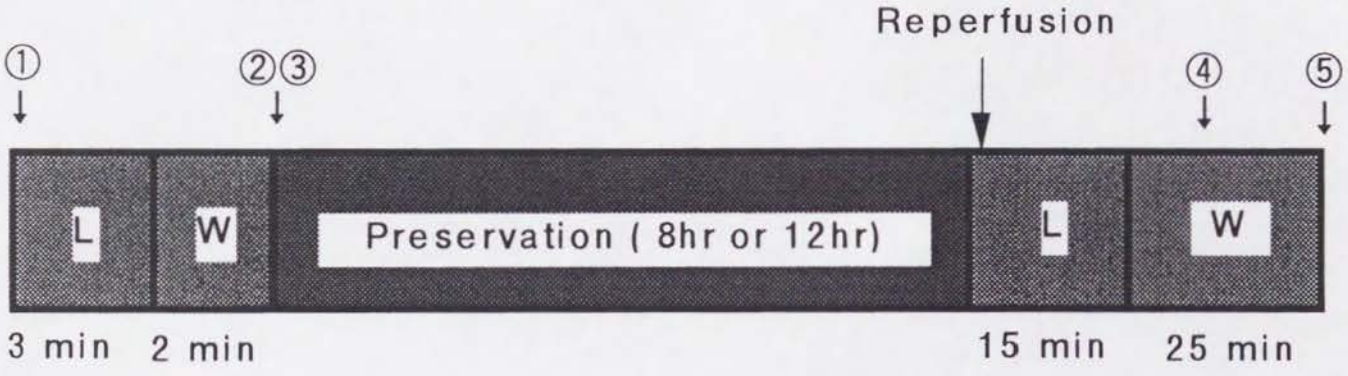
Reperfusion
①
②③
④
⑤
L
W
Preservation (8hr or 12hr)
L
W
3 min
2 min
15 min
25 min

Figure 1. Experimental Protocol. The hearts were excised and mounted on a Langendorff apparatus (①). They were arrested and stored for 8- or 12-hours, following measurement of baseline cardiac function in a W-mode (②, ③). Following cold storage, they were reperfused for 15 minutes on L-mode and 25 minutes on W-mode. The coronary perfusate was collected following 10 minutes of W-mode reperfusion to evaluate for lactate, CPK, and troponin-T (④). Cardiac functional recovery was measured at the end of W-mode reperfusion (⑤).

W: working mode perfusion, L: Langendorff mode perfusion, CPK: creatine phosphokinase

ROLE OF HYDROXYL RADICAL SCAVENGER (NICARAVEN) IN RECOVERY OF CARDIAC FUNCTION FOLLOWING PRESERVATION AND REPERFUSION

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Abstract

We investigated the efficacy in reducing myocardial preservation and reperfusion (P/R) injury of direct hydroxyl radical scavenging by nicaraven as compared with scavenging of both superoxide radicals and hydrogen peroxides by superoxide dismutase (SOD) and catalase (CAT), respectively. Isolated rat hearts were mounted on a Langendorff (L) apparatus to estimate the baseline aortic flow (AF), coronary flow (CF), cardiac output (CO), systolic pressure (SP), aortic mean pressure (MP), double product, and LV dp/dt. They were divided into 3 groups: Group 1, 12-hour storage in HTK solution; Groups 2, 12-hour storage in HTK solution containing 2.5 x 105 U/L SOD and 102 mg/L CAT; Group 3, 12-hour storage in HTK solution containing 10^{-3} M nicaraven. SOD and CAT, and nicaraven were administered intraperitoneally before harvesting. Hearts were stored in each preservation solution at 4°C , and then reperfused. Post-preservative function and concentrations of leaked enzymes were measured. The hearts were switched back to the L-mode and paced at 330 beats/min. CF following perfusion with Krebs-Henseleit bicarbonate buffer (KHB) solution containing 10^{-6}M 5-hydroxytryptamine (5-HT) or 10^{-5} M nitroglycerin (NTG) was expressed as a percentage of that perfused with drug-free KHB solution in each group. The myocardial water content also was measured. The recovery of CF, CO, SP, MP, and LV dp/dt was significantly greater in Group 3 than in Group 1. The recovery of CF was superior to that in Group 2 ($p<0.05$). There were no significant differences in the recovery of cardiac function, between Groups 1 and 2. 5-HT caused a decrease in CF in each group, however, CF in Group 3 was higher than that in Group 1 ($p<0.05$). NTG caused no significant differences among the groups. There were no significant differences in leaked enzymes and myocardial water content among the three groups. These results suggest that nicaraven protects against myocardial P/R injury through its hydroxyl radical scavenging activity, and that the therapy by oxygen-free radical scavengers might be preferentially toward inactivation of hydroxyl radicals rather than superoxide radicals and/or hydrogen peroxides.

Abbreviations: AF; aortic flow, CAT; catalase, CF; coronary flow, CO; cardiac output, CPK; creatine phosphokinase, EDRF; endothelium-derived relaxing factor, HR; heart rate, i.p.; intraperitoneally, 5-HT; 5-hydroxytryptamine, KHB; Krebs-Henseleit bicarbonate buffer, LA; left atrium, L; Langendorff, LV; left ventricle, MP; aortic mean pressure, NTG; nitroglycerin, P/R; preservation and reperfusion, RPP; rate pressure product, SOD; superoxide dismutase, SP; systoric pressure, SR; sarcoplastic reticulum, TAN; total adenine nucleotides, UW; University of Wisconsin, W; working

Introduction

Following the development of improved techniques, the preservation times of solid organs have been markedly extended. However, the generally accepted time limit for safe cold storage for clinical heart transplantation remains only 4-6 hours (1). It is thought that, during the ischemic period, there is an increase in the formation of free radicals that is greatly accelerated with the sudden reintroduction of oxygen at the onset of reperfusion; this leads to vascular damage and myocardial dysfunction (2). It has been suggested that free radicals, thus, are an important contributing factor in the development of reperfusion injuries (3,4).

The oxygen-derived free radicals and their metabolites include the superoxide radical, hydrogen peroxide, and the hydroxyl radical (5). The superoxide radicals and the hydrogen peroxide have a relatively low cellular reactivity in the absence of a transition metal such as iron (6,7). Instead, in the presence of iron, cellular injury is more likely related to the subsequent generation of the highly reactive hydroxyl radical via a superoxide-driven iron-catalyzed Fenton reaction (8), where superoxide radicals react directly with hydrogen peroxide to form hydroxyl radicals. Hydroxyl radicals are very reactive species that can interact with membranes and proteins, causing lipid peroxidation, protein denaturation, and altered membrane permeability (9).

Hydroxyl radical formation might be prevented by the simultaneous scavenging of both superoxide and hydrogen peroxide radicals. This is illustrated by the beneficial effects of the combined administration of superoxide dismutase (SOD) and the hydrogen peroxide scavenger, catalase (CAT), on reperfusion injury (10,11). However, it remains controversial whether an exogenous supply of these enzymes effectively reduces free radicals and improves cardiac function after ischemia and reperfusion (12,13).

Other investigators have reported that treatment with scavengers of hydroxyl radicals or agents that directly prevent their formation might reduce myocardial reperfusion injury (14-18). Some hydroxyl radical scavengers

previously have been tested with regard to their ability to reduce myocardial reperfusion injuries, however, their efficacy remains controversial (19-26). Nicaraven, 1, 2-bis(nicotinamide)-propane, has been reported to suppress lipid peroxidation by scavenging hydroxyl radicals and has shown a beneficial effect in the treatment of prolonged cerebral vasospasm after subarachnoid hemorrhage (27-30). Nicaraven, thus, might be able to reduce the development of myocardial and/or coronary vascular reperfusion injury following preservation.

The present study was designed by measuring cardiac function to evaluate the efficacy of direct hydroxyl radical scavenging by nicaraven, compared with scavenging of both superoxide radicals and hydrogen peroxides by SOD and CAT, respectively, in reducing myocardial preservation and reperfusion (P/R) injuries. In addition, we compared the protective effects of nicaraven on coronary vascular damage following P/R, with that in untreated-controls and those receiving combined treatment with both SOD and CAT (SOD & CAT).

Materials and Methods

Isolated rat heart preparation

Animal experimentation was performed in accordance with institutional guidelines of the Guide for the Care and Use of Laboratory Animals published by the U.S. Department of Health and Human Services. Male Wistar rats (250 to 350 g) were systemically heparinized [500 IU intraperitoneally (i.p.)], and anesthetized with sodium pentobarbital (65 mg/kg i. p.). The heart was excised and immediately immersed in Krebs-Henseleit bicarbonate buffer (KHB) solution, consisting of (mmol/L): NaCl (118), KCl (4.7), MgSO4 (1.2), KH2PO4 (1.2), CaCl2 (2.5), NaHCO3(25.0), and glucose (11.0) at 37°C. Then, it was mounted on a Langendorff apparatus (IPH-W, Labo Support, Osaka, Japan) via the aorta, and perfused with KHB solution at a constant pressure of 60 mmHg for 3 minutes in Langendorff mode (L-mode). KHB solution was filtered (48 μm), equilibrated with 95% O2 and 5% CO2, and maintained at 37°C. KHB perfusate in this circuit is not recycled. During

preparation, care was taken to cannulate the excised hearts rapidly to minimize the ischemic time. Following cannulation of the left atrium (LA) via the pulmonary vein, the heart was switched to working mode (W-mode) with an LA perfusion pressure of 10 mmHg and an afterload of 60 mmHg. Baseline function was determined following 2 minutes of W-mode. Measurements were as follows: aortic flow (AF), coronary flow (CF), cardiac output (CO), heart rate (HR), systolic pressure (SP), mean pressure (MP) and rate pressure product (RPP, HR × max SP).

Experimental protocol

Experimental protocol is shown in Figure 1. The hearts were divided into three groups (n=8 hearts per groups): Group 1, 12-hour storage in HTK solution; Group 2, 12-hour storage in HTK solution with 2.5 x 105 U/L SOD and 102 mg/L CAT; Group 3, 12-hour storage in HTK solution with 10-3 M nicaraven. 1.3 x 105 U SOD and 1.5 x 102 mg CAT, and 4 x 10-4 M nicaraven were administered intraperitoneally in Group 2 and 3, respectively, 15 minutes before harvesting. The hearts in all groups were arrested by administration of each preservation solution (50 mL/kg body weight at 4°C) via the aortic cannula at a pressure of 60 mmHg. Then, they were stored in each preservation solution (30mL) at 4°C. Following cold storage, they were mounted on a Langendorff apparatus again, and reperfused with KHB solution for 15 minutes on L-mode. Then, they were switched to W-mode. The coronary perfusate was collected following 10 minutes of W-mode reperfusion to evaluate for lactate, CPK, and troponin-T in each preservation group. Lactate, CPK, and troponin-T were determined as previously described (Ref 31, in press). Cardiac functional recovery of the stored heart was measured at the end of 25 minutes of W-mode reperfusion. Following evaluation of the stored heart, LV dp/dt (mmHg/sec) was measured by puncture via the left ventricular apex (Pressure Transducer, Nihon Kohden Corp., Tokyo, Japan), and the heart was switched back to L-mode and paced in an atrial mode at 330 beats/min (PGM-330, Labo Support, Osaka, Japan).

The hearts were, then, perfused with the drug-free KHB solution for 20 minutes for stabilization, during which CF was measured continuously. After stabilization, the hearts were perfused with KHB solution containing 10-6 M 5-hydroxytryptamine (5-HT) for 15 minutes and CF was recorded for the last 2 minutes of perfusion. The coronary circulation was washed with the drug-free KHB for the next 20 minutes to reestablish the basal CF. The hearts subsequently were perfused with KHB solution containing 10-5 M nitroglycerin (NTG) for 15 minutes and CF was measured for the last 2 minutes of perfusion. The increase in CF after perfusion with KHB solution containing 5-HT or NTG was calculated and expressed as a percentage of that of the heart perfused with drug-free KHB solution in each group.

A ventricular specimen was weighed immediately, dried at 80°C to constant weight, and reweighed following 24 hours. Water content was computed using the following formula: water contents (%) = (wet weight - dry weight)/ wet weight × 100.

Chemicals and reagents

Nicaraven, 1, 2-bis(nicotinamide)-propane was obtained from Chuugai Pharmaceutical. Co. LTD, Japan, 5-hydroxytryptamine (5-HT) from Wako Pure Chemical Industries, LTD, Japan, nitroglycerin (NTG) from Nippon Kayaku Co., LTD, Japan, SOD and CAT from Sigma Chemical, Co.LTD, USA.

Statistical analysis

All results are expressed as the means ± S.E. Statistical evaluation of the data was performed by one-way ANOVA and Scheffe's F-test. A p value of less than 0.05 was considered statistically significant.

Results

Baseline cardiac function

The mean values of baseline cardiac function (n=24), measured prior to

preservation, were as follows; 46.4 ± 2.9 ml/min of AF, 15.4 ± 1.1 ml/min of CF, 61.3 ± 3.4 ml/min of CO, 238.8 ± 14.6 beats/min of HR, 112.3 ± 4.6 mmHg of SP, 55.3 ±0.6 mmHg of MP and 25634 ± 421 mm Hg beats/min of RPP.

Cardiac functional recovery

Table 1. shows the recovery of hemodynamic data on the heart for 12 hours of preservation [(A), absolute values of post-preservative cardiac function; (B), percentage values to the pre-preservative baseline function).The recovery of CF, CO, SP, MP, and LV dp/dt in the nicaraven group (Group 3) was significantly greater than that in the control group (Group 1). The recovery of CF in the nicaraven group also was superior to that in the SOD & CAT group (Group 2) ($p<0.05$). The recovery of CO in the nicaraven group showed a tendency to increase, compared with those in the SOD & CAT group ($p=0.07$).

After the first 20-minute stabilization period, CF perfused with drug-free KHB solution was 5.1±0.6 ml/min in Group 1, 5.5 ± 0.4 ml/min in Group 2, and 5.8 ±0.4 ml/min in Group 3. After perfusion with KHB solution containing 5-HT, CF was 4.1±0.5 ml/min in Group 1, 4.6 ± 0.4 ml/min in Group 2, and 5.4 ±0.3 ml/min in Group 3. CF in Group 3 was higher than that in Group 1 ($p<0.05$)(Table 2). In addition, 5-HT caused a decrease in CF(Group 1, -20.5 ± 3.0 %; Group 2, -15.4 ± 4.8 %; Group 3, -5.9 ± 3.8 %) (Figure 2), compared with that of the heart perfused with drug-free KHB solution. However, this decrease of CF was less in the nicaraven group than in the control group ($p<0.05$)(Figure 2).

After the next 20-minute stabilization period, CF perfused with drug-free KHB solution was 4.8±0.5 ml/min in Group 1, 5.3 ± 0.3 ml/min in Group 2, and 5.6±0.3 ml/min in Group 3. After perfusion with KHB solution containing NTG, CF was 5.2±0.4 ml/min in Group 1, 5.3 ± 0.4 ml/min in Group 2, and 5.8 ±0.4 ml/min in Group 3. NTG caused a little increase in CF (Group 1, 8.8 ± 6.7 %; Group 2, –0.6 ± 1.8 %; Group 3, 3.4 ± 1.3 %) (Figure 2), compared

with that of the heart perfused with drug-free KHB solution. However, there were no significant differences in CF among the groups.

CPK, Lactate and Troponin-T leakage

The values of CPK leakage in Group 1, 2, and 3 were 0.1±0.0 IU/min/g, 0.1±0.1 IU/min/g, and 0.2±0.1 IU/min/g, respectively. The values of lactate leakage were 7.8±0.7 mg/min/g, 10.4±2.1 mg/min/g, and 9.6±0.7 mg/min/g, respectively. The values of troponin-T leakage were12.3±4.0 ng/min/g , 12.9±3.7 ng/min/g, and 16.9±2.8 mg/min/g, respectively. There were no significant differences in CPK, troponin-T, and lactate leakage among the three groups.

Water content

The tissue water content was 83.5 ± 0.4 % in Group 1, 84.6 ± 1.5 % in Group 2, and 83.1 ± 1.3 % in Group 3. There were no statistically significant differences among the groups.

Discussion

The most important hypotheses explaining the cellular events involved in reperfusion damage are oxygen-derived free radical damage and calcium overload (2). Free radicals, such as the superoxide radical, hydrogen peroxide, and hydroxyl radical, probably exert their damaging effects by disrupting membrane lipids or membrane-bound proteins. These impaired cell membranes cause an increase in intracellular free calcium which may lead to reperfusion injuries such as reperfusion arrythmias, vascular damage and no-reflow, and myocardial stunning (2,9). Xanthine oxidase may serve as the initial source of free radical generation in post-ischemic reperfusion injuries. During ischemia, ATP is degraded to its purine bases (e.g. hypoxanthine). At reperfusion, oxygen becomes available, suddenly and in exess, and the oxidation of hypoxanthine and xanthine proceeds rapidly, generating a burst of superoxide radicals and hydrogen peroxides which may overcome the

endogenous antioxidant system (32). Therefore, conditions are created in which the highly reactive hydroxyl radicals can be produced (33).

Hydroxyl radical formation can be prevented by the simultaneous scavenging of both superoxide and hydrogen peroxide radicals (10,11). Alternatively, hydroxyl radicals might be susceptible to direct scavenging by hydroxyl radical scavengers (14-18). Whereas, superoxide dismutase (SOD) and catalase (CAT) metabolize superoxide radical and hydrogen peroxide, respectively, to benign metabolites (10,11), nicaraven may directly scavenge hydroxyl radicals (27-30). In this study, we investigated the protective effects of administering free radical scavengers following preservation and reperfusion (P/R). We found better recovery of cardiac function in the nicaraven group than that in the non-treated control and SOD & CAT-treated groups. These results suggest that nicaraven may reduce myocardial P/R injury more effectively than combined treatment with both SOD and CAT.

In the isolated rat heart, 5-hydroxytryptamine (5-HT) may cause coronary vasodilation by the release of endothelium-derived relaxing factor (EDRF). However, when the release of EDRF is blocked or the endothelium is damaged, the vasoconstrictive effect of 5-HT on the coronary vasculature is unmasked (34). Therefore, 5-HT leads to an increase in coronary flow (CF) in the absence of coronary endothelial damage. In this study, 5-HT caused a decrease in CF, from which it may be postulated that the coronary endothelium was damaged during P/R. Examination of cardiac function in the working mode revealed better recovery of CF in the nicaraven-treated group than that in SOD & CAT-treated and non-treated control groups. Nicaraven, thus, might have exerted a protective effect on the coronary endothelium that led to an attenuation of the decrease in CF induced by 5-HT following P/R.

Halliwell et al. (22) have reported that the amount of free iron is limited or that iron and hydrogen peroxide are present in different compartments. Therefore, hydrogen peroxide may not exert a damaging effect since the hydroxyl radical is not produced when iron and hydrogen peroxide do not come contact. The superoxide radical and hydrogen peroxide may not be

directly toxic, but, instead, cellular injury might be more likely related to the subsequent generation of the more highly reactive hydroxyl radical (6-9). Thus, therapy with oxygen-free radical scavengers should be directed toward inactivation of hydroxyl radicals, rather than the superoxide radical and/or hydrogen peroxide. It has been reported that SOD has an extremely short-half-life which may limit its efficacy since considerably more time may be required until an effective tissue concentration is reached (35). It might be partly attributable to this reason that the recovery of cardiac function in the SOD/CAT-treated group did not improve as much as that in the non-treated control group. Weglicki et al. (36,37) have reported that scavengers such as SOD and CAT may fail to scavenge free radicals when they are generated within the membrane. Nicaraven, which is not only hydrophilic but also lipophilic, might be able to contact to and/or permeate a cell membrane and scavenge hydroxyl radicals formed at the cell membrane (27-30,37). This may explain the improvement in cardiac function in the nicaraven-treated group in this study.

We have previously reported that HTK solution is superior to University of Wisconsin (UW) solution for cardiac preservation following 8-hour storage (in press). Therefore, in this study, we used HTK solution as a basal preservation solution and examined cardiac functions following a longer 12-hour period of preservation. In our isolated heart model, neutrophils and platelets were absent from the perfusion solution, however, other sources of free radical production, such as mitochondria, catecholamine oxidation, arachidonic acid metabolism, and xathine oxidase system could have produced free radicals (38). Furthermore, the present study did not investigate the efficacy of administering free radical scavengers during reperfusion. The results of this study may indicate that for optimal sustained protection, prolonged administration of free radical scavengers (up to several hours) may be revealed in the setting of a blood-perfused transplanted heart.

In conclusion, nicaraven, a hydroxyl radical scavenger attenuates P/R injury and improves the recovery of cardiac function. This may lead to a safe

extension of the heart preservation time and improve morbidity and mortality rates for patients undergoing cardiac transplantation. Future studies of nicaraven pretreatment, combined with nicaraven administration during reperfusion, in the setting of prolonged hypothermic storage may be warranted to determine if further improvements in cardiac function can be obtained.

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TABLE 1. (A) Cardiac Functional Recovery

Group	Time (hour)	AF (ml/min)	CF (ml/min)	CO (ml/min)	SP (mmHg)	MP (mmHg)	RPP ($x10^3$)	LVdp/dt
1.	12	1.9±0.9	4.5±1.3	6.6±2.4	44.1±8.8	27.5±5.5	7.3±1.6	539±142
2.	12	2.3±0.4	6.0±0.5	9.1±0.8	58.3±4.4	41.3±2.2	10.4±2.1	757±84
3.	12	6.0±2.3	9.5±0.8*#	15.6±3.1*	89.2±3.0*	44.6±1.4*	11.2±1.6	960±48*

(B) Cardiac Functional Recovery (%)

Group	Time (hour)	AF (%)	CF (%)	CO (%)	SP (%)	MP (%)	RPP (%)
1.	12	3.9±2.3	29.7±9.2	11.7±3.9	37.9±8.7	52.9±10.5	28.1±6.4
2.	12	5.1±0.9	43.0±3.1	15.0±1.4	53.4±3.7	75.9±3.9	40.8±8.0
3.	12	13.0±5.4	62.8±5.6*#	25.6±5.1*	61.0±3.9*	80.6±2.8*	42.7±6.0

n=8 hearts per groups. Data are expressed as mean±S.E. The hemodynamics of the pre-preservative hearts were: AF(46.4±2.9 ml/min), CF(15.4±1.1 ml/min), CO(61.3±3.4 ml/min), HR(239±15 beats/min), SP(112.3±4.6 mmHg), MP(55.3±0.6 mmHg) and RPP(25634±421 mmHg beats/min).

*$p<0.05$ vs Group 1, #$p<0.05$ vs Group 2

Group1, control group; Group2, SOD&CAT group; Group3, nicaraven group.

SOD, superoxide dismutase $2.5x10^5$ U/L; CAT, catalase 10^2 mg/L; nicaraven, nicaraver 10^{-3} M.

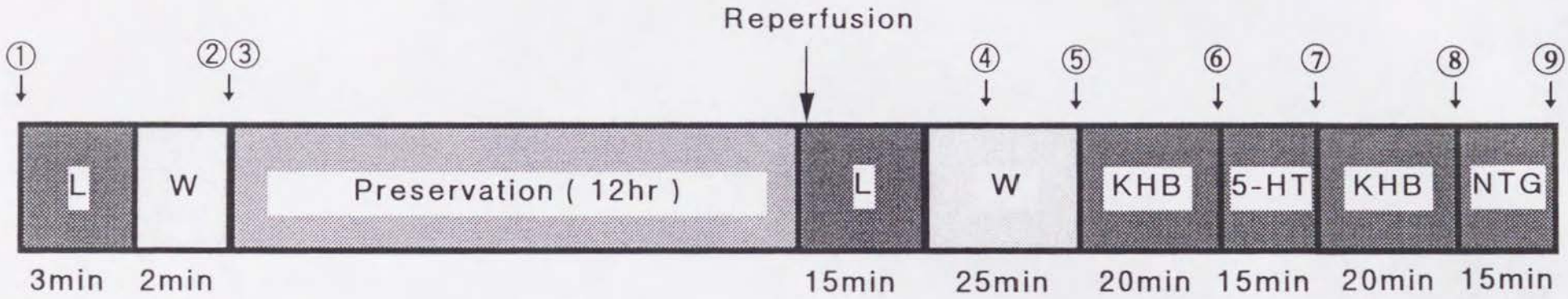
AF, aortic flow; CF, coronary flow; CO, cardiac output; HR, heart rate;

SP, systolic pressure; MP, aortic mean pressure; RPP, rate pressure product (mmHg beats/min)

Table 2. Coronary flow (ml/min)

Group	KHB(20 min) →	5-HT(15 min) →	KHB(20 min) →	NTG(15 min)
1.control	5.1±0.6	4.1±0.5	4.8±0.5	5.2±0.4
2. SOD&CAT	5.5±0.4	4.6±0.4	5.3±0.3	5.3±0.4
3. nicaraven	5.8±0.4	5.4±0.3*	5.6±0.3	5.8±0.4

n=8 hearts per groups. Data are expressed as mean±SE.
*Statistically significant differences ($p<0.05$)
SOD, superoxide dismutase 2.5×10^5 U/L; CAT, catalase 10^2 mg/L; nicaraven, nicaraven 10^{-3} M.
KHB, Krebs-Henseleit bicarbonate buffer; 5-HT, 5-hydroxytryptamine; NTG, nitroglycerin.


Reperfusion
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20min
15min

FIGURE 1. Experimental Protocol. The hearts were excised and mounted on a Langendorff apparatus (①). They were arrested and stored for 12-hours, following measurement of baseline cardiac function in a W-mode (②,③). Following cold storage, they were reperfused for 15 minutes on L-mode and 25 minutes on W-mode. The coronary perfusates were collected, following 10 minutes of W-mode reperfusion to evaluate for lactate, CPK, and troponin-T (④). Cardiac functional recovery was measured at the end of W-mode reperfusion and the hearts were switched back to L-mode and paced in an atrial mode at 330 beats/ min (⑤). The coronary circulation was washed with the drug-free KHB for 20 minutes to establish the basal coronary flow (CF) (⑥). After the 20-minute stabilization period, the hearts were perfused with KHB solution containing 10^{-6} M 5-HT for 15 minutes and CF was recorded for the last 2 minutes of perfusion (⑦). The coronary circulation was washed with the drug-free KHB solution again for the next 20 minutes to reestablish the basal CF (⑧). The hearts subsequently were perfused with KHB solution containing 10^{-5} M NTG for 15 minutes and the CF was measured for the last 2 minutes of perfusion (⑨). W: working mode perfusion, L: Langendorff mode perfusion, CPK: creatine phosphokinase, KHB: Krebs-Henseleit bicarbonate buffer , 5-HT: 5-hydroxytryptamine, NTG: nitroglycerin, CF: coronary flow

Coronary flow reaction to 5-HT and NTG (%)

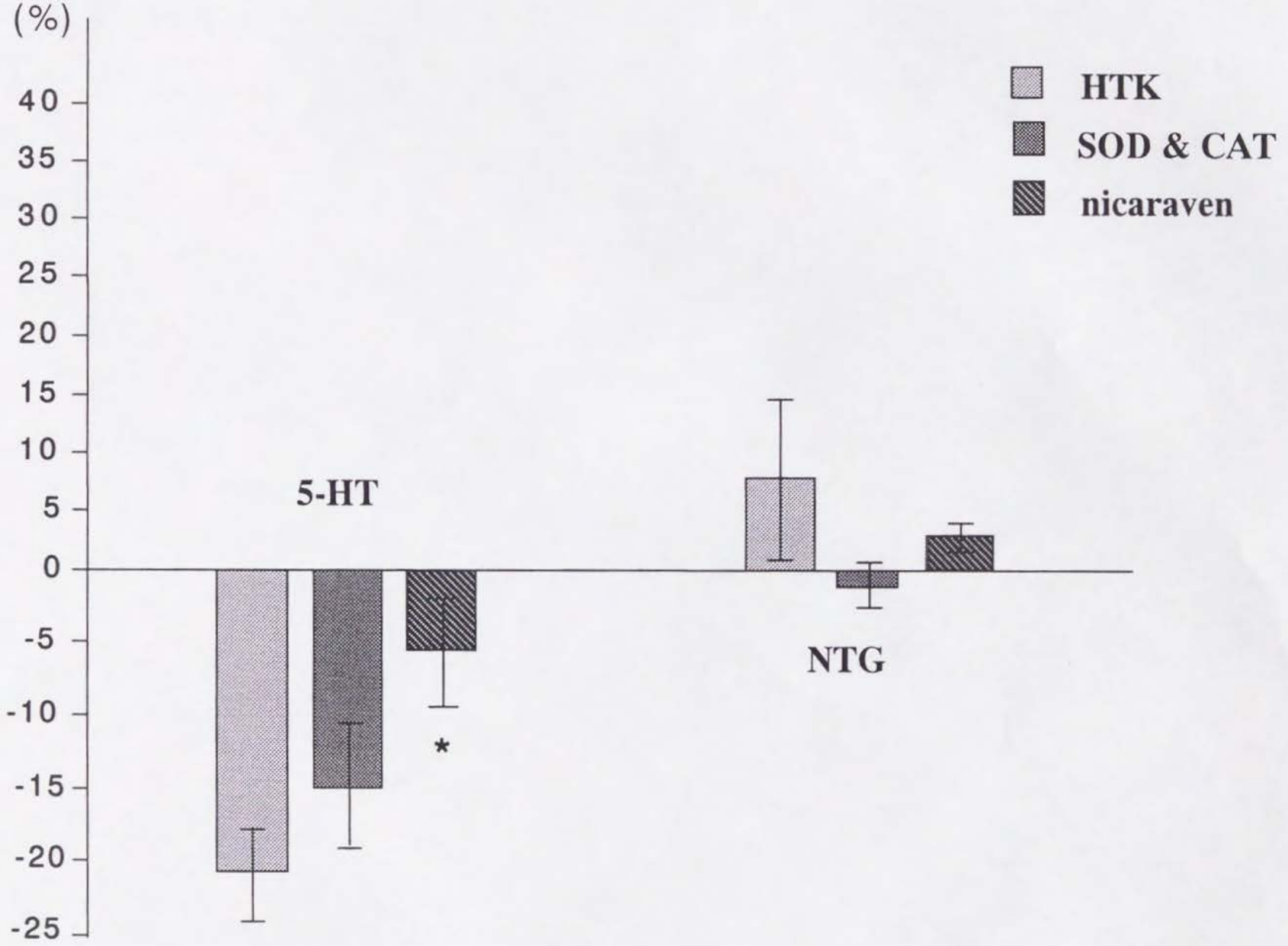

FIGURE 2. The coronary flow (CF) reaction to 5-HT and NTG, following preservation and reperfusion. CF after perfused with KHB solution containing 5-HT and NTG was calculated and expressed as a percentage of that of the heart perfused with drug-free KHB solution in each group. After 20-minute stabilization period, CF perfused with drug-free KHB solution in Group 1, 2, and 3 was 5.1±0.6ml/min, 5.5±0.4ml/min, 5.8±0.4ml/min, respectively. 5-HT caused a decrease in CF, compared with that of the heart perfused with drug-free KHB solution. However, this decrease was less in Group 3 than in Group 1 (*$p<0.05$). After the next 20-minute stabilization period, CF perfused with drug-free KHB solution in Group 1, 2, and 3 was 4.8±0.5ml/min, 5.3±0.3ml/min, 5.6±0.3ml/min, respectively. NTG caused no significant differences in CF among the groups.
Group 1; control group, Group 2; SOD & CAT group, Group 1; nicaraven group.

SOD, superoxide dismutase 2.5 $x10^5$ U/L; CAT, catalase 10^2 mg/L; nicaraven, nicaraven 10^{-3} M.
KHB, Krebs-Henseleit bicarbonate buffer